Arch. Environ. Contam. Toxicol. 32, 141–145 (1997)

ARCHIVES OF
Environmental
Contamination
and Toxicology

Metabolites of Arsenic Induced Tetraploids and Mitotic Arrest in Cultured Cells

N. Eguchi, K. Kuroda, G. Endo

Department of Preventive Medicine and Environmental Health, Osaka City University Medical School, 1-4-54, Asahi-machi, Abeno-ku, Osaka 545, Japan

Received: 9 April 1996/Revised: 15 June 1996

Abstract. The toxic effects of arsenic compounds on cell division were studied, using Chinese hamster V79 cells. Seven arsenic compounds were tested. Inorganic arsenic compounds (arsenite and arsenate), which have been found in drinking water, inhibited cell growth at very low concentrations. Monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), and trimethylarsine oxide (TMAO), which are methylated metabolites of inorganic arsenics, were less cytotoxic than the inorganic arsenics themselves. The cytotoxicity of the three methylated metabolites decreased as the number of methyl groups increased. Arsenobetaine (AsBe) and arsenocholine (AsC), which have been found in some marine products, did not show any cytotoxicity. Three methylated metabolites; MMA, DMA and TMAO induced mitotic arrest. Tetraploidy production was observed in cells exposed to DMA or TMAO. Arsenite, arsenate, AsBe and AsC did not induce mitotic arrest or tetraploids. These results suggest that MMA, DMA and TMAO exert some effect on cell division in metaphase and may thereby give some clue as to the carcinogenic mechanism of arsenic.

Arsenic is a human carcinogen. However, the ultimate carcinogen of arsenic compounds is unknown (IARC 1987). Inorganic arsenic compounds found in drinking water are associated with increased risk of lung cancer and skin cancer (IARC 1980). Recently, exposure of arsenic to semiconductor workers has been regarded to be dangerous (Fowler and Silbergeld 1989). It is well known that arsenite and arsenate act as acute toxins. Yager *et al.* (1993) demonstrated that in cultured cells arsenite acts synergistically with 1,3-butadiene diepoxide in the induction of chromosomal aberrations. However, inorganic arsenic compounds alone did not induce transformations (Jacobson-Kram and Montalbano 1985). Similarly, in animal studies, these compounds alone are not directly tumorigenic.

Dimethylarsinic acid (DMA) is a major form of arsenic in the environment and has been used as a general herbicide or pesticide for many years (Wagner and Weswig 1974). In most mammals, including humans, DMA is one of the major methylated metabolites of ingested arsenics (Vahter *et al.* 1980), which eliminated through the kidneys, to be excreted in the urine. Despite the negative results yielded by inorganic arsenics in some mutagenicity assays, recent in vivo findings have revealed that DMA induced urinary bladder cancer in rats after pretreatment with *N*-butyl-*N*-(4-hydroxybutyl) nitrosamine, and acted as a promoter of kidney, liver and thyroid gland carcinogenesis in rats (Yamamoto *et al.* 1995). Endo *et al.* (1992) reported that DMA induced aneuploidy, such as tetraploid formation and arrests mitosis.

Many investigators have proposed DMA to be a potent clastogenic agent, causing gene amplification and acting as a tumor promoter (Lee *et al.* 1988; Beckman and Nordenson 1986). Apart from DMA, other methylated metabolites of ingested arsenic are monomethylarsonic acid (MMA) and trimethylarsine oxide (TMAO). It would be useful to study whether MMA and TMAO induced tetraploidy and arrested mitosis. Arsenobetaine (AsBe) and arsenocholine (AsC) are found in one some marine products. We investigated the effects on the cell division and the cytotoxicity of seven arsenic compounds which are arsenite, arsenate, MMA, DMA, TMAO, AsBe and AsC (Figure 1).

Materials and Methods

Arsenic compounds were purchased from Tri-Chemical, Kanagawa, Japan. Sodium arsenite (Na_2HAsO_3) (purity > 99.9999%), sodium arsenate (Na_2HAsO_4) (purity > 99.99%), AsBe (purity > 99%), AsC (purity unknown), MMA (purity > 99.99%), DMA (purity > 99.99%), and TMAO (purity > 99%) were dissolved in distilled water, and added directly to the culture medium. The final concentrations of arsenic compounds in the cultures ranged from 0.01 mg/ml to 10 mg/ml. 5-Bromodeoxyuridine (BrdU, 1 μg/ml), colcemid and Hoechst 33258 were purchased from Wako Pure Chemicals, Osaka, Japan. Giemsa's solution was obtained from Merck, Darmstadt, Germany. V79 cells, which originated from Chinese hamster lung, were purchased from ATCC, Rockville, Maryland. Eagle's MEM and fetal bovine serum were obtained from ICN Biochemicals, Costa Mesa, California. Trypsin was purchased from Difco, Detroit, Michigan.

V79 cells were added to Eagle's MEM supplemented with 7% heat-inactivated fetal bovine serum, penicillin (100 units/ml), streptomycin (100 μg/ml) and BrdU (1 μg/ml). The cultures were incubated in the dark, at 37°C, for 28 h in a 5% CO_2 atmosphere. Colcemid was added, to a final concentration of 0.1 μg/ml, into the culture medium for the last 2 h of incubation. Cells were harvested with trypsin solution, treated with a hypotonic solution of 0.075 M KCl and fixed on glass microscope slides with methanol-acetic acid (3:1). The metaphase figures were stained the next day with 0.1 μg/ml Hoechst 33258, irradiated with a mercury lamp and stained with 2% Giemsa's solution.

Correspondence to: N. Eguchi

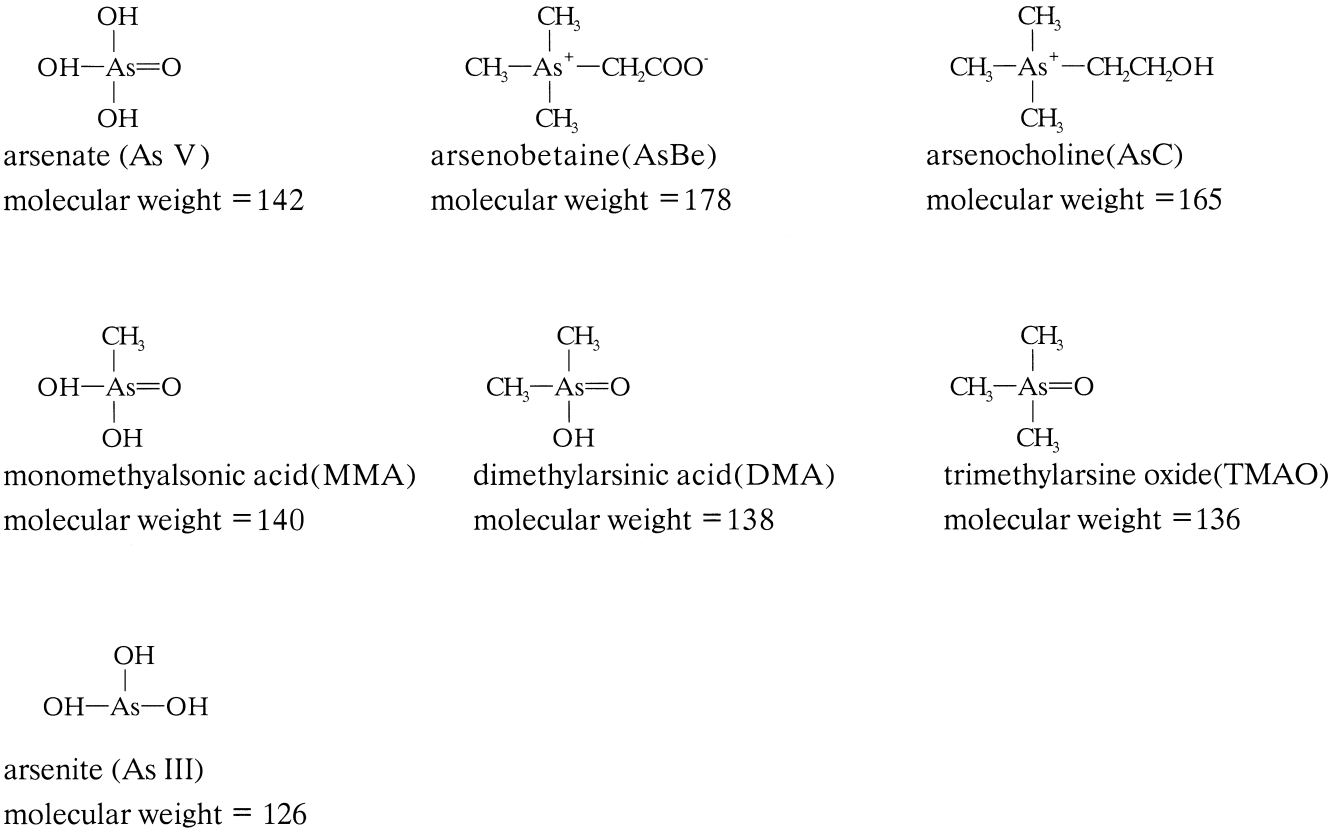

Fig. 1. Arsenic compounds

Statistical differences in means were determined by Student's *t*-test. All p values were two-tailed, and a p value <0.05 was considered significant.

Results

The doubling time of the V79 cells was about 18 h. The number of chromosomes per cell ranged from 20 to 25. About 90% of the cells had 22 chromosomes. Hence, metaphase figures with 40 to 50 chromosomes were considered tetraploids. The rate of spontaneous tetraploid formation was about 2% (Endo *et al.* 1992). In twice-divided metaphase figures, newly synthesized sister chromatids and old chromatids were completely differentially stained, while, in once-divided metaphases, they were not differentially stained. In thrice-or-more divided metaphases, they were partially differentially stained.

The concentrations of arsenic compounds that inhibited growth of treated cells by 50%, as compared with untreated cells, are shown in Table 1. Among the chemicals administered, arsenite was found to have the highest toxicity at a concentration which was 1 to 2 fold lesser than that of arsenate. MMA and DMA were twofold less toxic than arsenate, while TMAO exhibited least toxicity. AsBe and AsC were not toxic even at a high concentration of 20 mg/ml. The rank order of cytotoxicity has been summarized as follows: arsenite > arsenate ≫ MMA > DMA > TMAO, AsBe and AsC.

MMA, DMA and TMAO induced excess mitotic arrest (Table 2, Table 3, Figure 2, Figure 3). The concentrations of MMA that induced mitotic arrest were lower than those of DMA and TMAO. AsBe and AsC did not induce mitotic arrest (Figure 3). Metaphase figures were not observed in cells exposed to arsenite and arsenate, therefore, it appears that these inorganic arsenics do not induce mitotic arrest.

Table 1. Cytotoxicity of arsenic compounds in V79 cells

Arsenic Compounds	50%-Growth- Inhibition Dose (mg/ml)
Sodium arsenite	0.0008
Sodium arsenate	0.04
Methylarsonic acid (MMA)	0.7
Dimethylarsinic acid (DMA)	3.3
Trimethylarsine oxide (TMAO)	>10
Arsenobetaine (AsBe)	>10
Arsenocholine (AsC)	>10

As shown in Figure 3, DMA and TMAO induced tetraploids in a dose-dependent manner; that is to say the number of tetraploids increased with increase in concentration of these substances. No tetraploids were observed in cells exposed to MMA.

Discussion

The order of toxicity potential in rats from the data of NIOSH (1976) was as follows: arsenite > arsenate > MMA > DMA > TMAO. In the in vitro and in vivo findings, the same order of cytotoxicity were obtained. MMA has one methyl group and

Table 2. Mitotic index, tetraploids index

Arsenic Compounds (mg/ml) (mM)	Total Number of Cells	Number of Mitotic Cells	Metaphase Division			Tetraploids Index[b]
			Once	Twice	Thrice or More Times	
None	1000	36	1 (2.8%)	25 (69.4%)	10 (27.8%)	0%
Water	1000	43	1 (2.3%)	28 (65.1%)	14 (32.6%)	3.5%
Arsenobetaine (1 mg/ml = 5.6 mM)	1000	24	1 (4.2%)	20 (83.3%)	3 (12.5%)	0%
Arsenocholine (1 mg/ml = 6.1 mM)	1000	43	4 (9.3%)	32 (74.4%)	7 (16.3%)	3.1%
Sodium arsenite (0.0005 mg/ml = 0.0038 mM)	1000	17	7 (41.2%)	10 (58.8%)	0 (0.0%)	0%
Sodium arsenate (0.03 mg/ml = 0.18 mM)	1000	20	2 (10.0%)	17 (85.0%)	1 (5.0%)	0%
Monomethylarsonic acid (0.5 mg/ml = 3.6 mM)	1000	62[a]	62 (100%)	0 (0.0%)	0 (0.0%)	0%
Dimethylarsinic acid (1 mg/ml = 7.2 mM)	1000	114[a]	72 (63.2%)	38 (33.3%)	4 (3.5%)	47.3%[a]
Trimethylarsine oxide (1 mg/ml = 7.4 mM)	1000	51	3 (5.9%)	40 (78.4%)	8 (15.7%)	22.5%[a]

Arsenate in concentrations of 0.03–0.06 mg/ml inhibited cell proliferation and at a concentration of 0.1 mg/ml killed the cells

[a] Significant difference from the group without arsenic at $p < 0.05$

[b] (Number of tetraploids)/(number of twice divided metaphase score)

Table 3. Mitotic index and tetraploids index vs Trimethylarsine oxide (TMAO) concentrations

Arsenic Compounds (mg/ml)	Total Number of Cells	Number of Mitotic Cells	Metaphase Division			Tetraploids Index[b]
			Once	Twice	Thrice or More Times	
None	1000	36	1	25	10	0%
Water	1000	43	1	28	14	3.5%
0.05	1000	37	3	31	3	0%
0.1	1000	21	2	18	1	0%
0.2	1000	51	1	40	10	4.8%
0.5	1000	48	1	40	7	7.1%
1	1000	51	3	40	8	22.5%[a]
2	1000	39	1	37	1	16.5%[a]
4	1000	93[a]	6	85	2	35.0%[a]
10	1000	320[a]	21	299	0	47.6%[a]

[a] Significant difference from the group without arsenic at $p < 0.05$

[b] (Number of tetraploids)/(number of twice divided metaphase score)

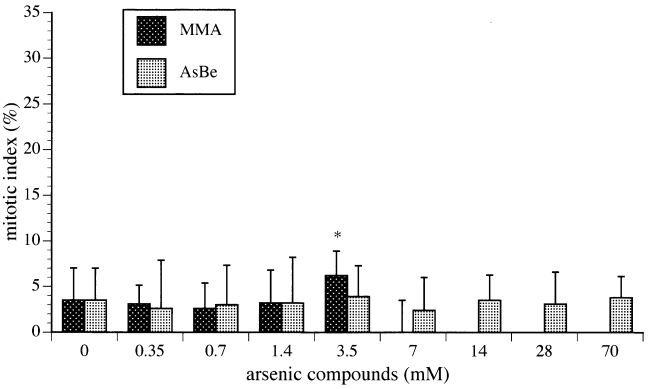

Fig. 2. The rate of cells in M phase vs MMA and AsBe concentrations. Values are means ± SE. *Significant difference from the group without arsenic at $p < 0.05$

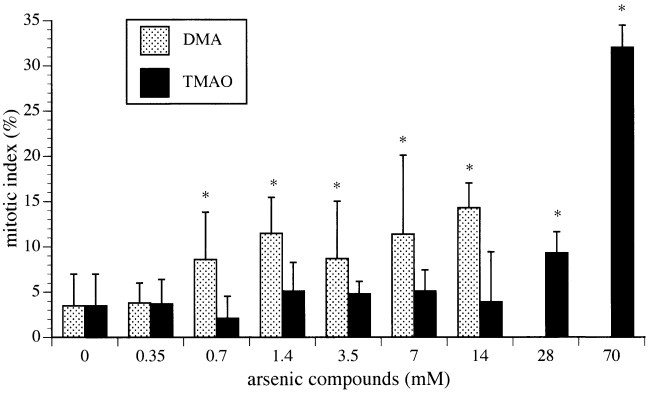

Fig. 3. The rate of cells in M phase vs DMA and TMAO concentrations. Values are means ± SE. *Significant difference from the group without arsenic at $p < 0.05$

two hydroxyl groups, and DMA has two methyl groups and one hydroxyl group (Figure 1). TMAO has three methyl groups and no hydroxyl group. The low reactivity of the methyl group than the hydroxyl group may be the reason for its low toxicity. This could be the rationale for the rank order of cytotoxicity: MMA > DMA > TMAO.

Ochi et al. (1994) reported the cytotoxicity of aresnics *in vitro* with the following order of ranking: arsenite > arsenate > DMA > MMA > TMAO. There may be two reasons why the order of MMA and DMA in our data differed from that in their data. One reason could be the difference in purity of arsenic compounds. In this study, MMA and DMA, with purities greater than 99.99%, obtained from Tri Chemical, Kanagawa, Japan were used. Our 50%-growth-inhibition dose for DMA was equal to that previously reported by Ochi et al. (1994) but the value for MMA was 10 fold lower than the reported data. Hence the rank order of cytotoxicity became: MMA > DMA. The previous experiments produced the same results when we used MMA and DMA with unknown purity from Wako Pure Chemicals, Osaka, Japan. The concentration of MMA used in the previous work was measured by ion chromatography with the inductively coupled plasma mass spectrometry (IC-ICP-MS) method and proved to be lower than that used in the present study (Inoue *et al.* 1994; Kawabata *et al.* 1994). It has been reported that DMA used in the previous study contained inorganic arsenic (Yamauchi *et al.* 1984). These contaminants, such as inorganic arsenic, have presumably influenced the potential of MMA and DMA cytotoxicity. The other reason could be the difference in cell lines: V79 (Chinese hamster lung) and BALB/c 3T3 (mouse fibroblast) cells.

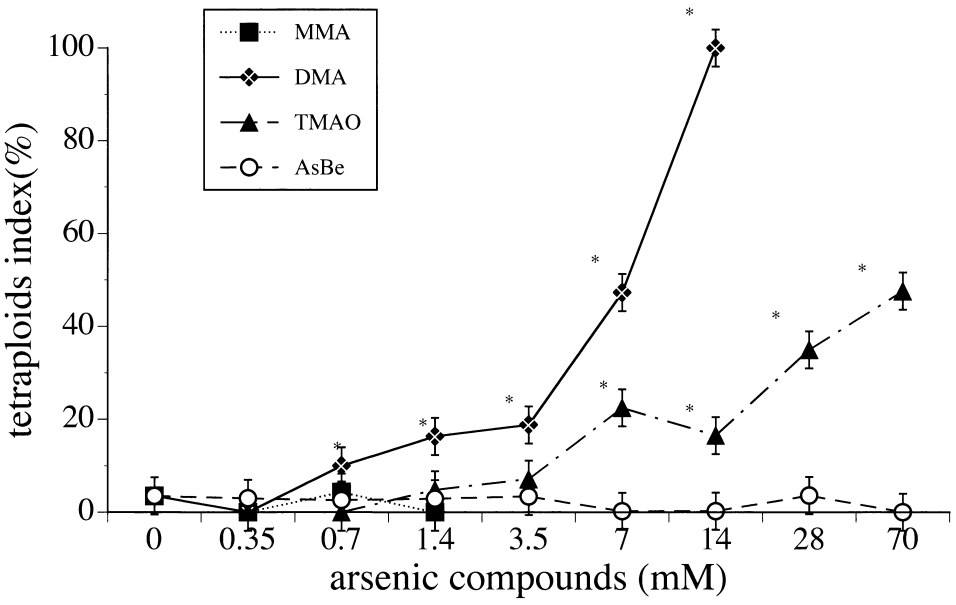

Fig. 4. The incidence of tetraploid formation in several concentrations of arsenic compounds. Tetraploids index = number of tetraploids/ number of twice divided metaphase figures. Values are means ± SE. *Significant difference from the group without arsenic at $p < 0.05$

Table 4. The actions of arsenics

	Arsenite	Arsenate	MMA	DMA	TMAO	AsBe and AsC
Cytotoxicity	++++	+++	++	+	±	−
Induction of mitotic arrest	−	−	+++	++	+	−
Induction of tetraploids	−	−	−	++	++	−

MMA = Monomethylarsonic acid
DMA = Dimethylarsinic acid
TMAO = Trimethylarsine oxicide
AsBe = Arsenobetaine
AsC = Arsenocholine

As shown in Figures 2 and 3, MMA, DMA and TMAO induced mitotic arrest. DMA and TMAO induced tetraploids. According to these results, the rank orders of seven arsenic compounds are listed with regard to cytotoxicity, induction of mitotic arrest and induction of tetraploids (Table 4).

MMA, DMA, and TMAO arrested the cell cycle in the M (mitosis) stage. No suggestions have been made regarding the mechanisms involving the arrest of mitosis by the arsenics in the literature to date. In the M stage of the cell cycle, tubulin polymerization and mitotic spindle formation is observed. The chromosomes attach to the mitotic spindles and microtubule eventually align the chromosomes in one place halfway between the spindle poles. Each chromatid then separates and is pulled toward the spindle poles. Biochemical substances, such as cyclin, mitosis promoting factor (MPF), protein phosphatase and topoisomerase II, are known to influence the M phase. The cell cycle is regulated by breaks that can stop the cycle at specific checkpoints (Nurse 1990). Antimitotic drugs which act in various points were known. Colchicine, colcemid, vinblastine and vincristine act on microtubules, and induce mitotic arrest and tetraploids (Rieder and Palazzo 1992). Each molecule of colchicine binds tightly to one tubulin molecule and prevents its polymerization. Vinblastine and vincristine, which are widely used in the treatment of cancer, temporarily disrupt spindle microtubules. The inactivation of MPF that signals the metaphase-to-anaphase transition is blocked so long as the spindle is disassembled and mitotic arrest is thus induced (Hoyt *et al.* 1991). Etoposide which inhibits topoisomerase II disturbs chromosome condensation and induces mitotic arrest (Roberge *et al.* 1990), while Okadaic acid which inhibits protein phosphatase also induces mitotic arrest (Felix *et al.* 1990).

Regarding the mechanism of induction of tetraploids, Vega *et al.* (1995) suggested that arsenite induced incomplete mitotic arrest, which lead to tetraploid formation. Staurosporine, a microbial alkaloid and a strong inhibitor of protein kinase, also induced tetraploids (Bruno *et al.* 1992). Staurosporine influenced cyclin B and arrested cell cycle in the G_2 (gap2) stage (Gong *et al.* 1993), while arsenics seemed to exert its influence in the mitotic stage of the cell cycle. Therefore, the mechanism by which arsenics induced tetraploids differed from that of staurosporine.

AsBe and AsC did not show cytotoxicity and did not cause mitotic arrest or tetraploid formation. Kaise *et al.* (1992) and Vahter *et al.* (1983) demonstrated that AsBe and AsC were excreted without being metabolized in vivo. Both these compounds have three methyl groups and so are biochemically stable. It is because of these features that these substances exhibited absence of cytotoxicity or inhibition of cell growth.

Arsenite and arsenate did not cause mitotic arrest or tetraploid formation in this study. These substances were far more cytotoxic than organoarsenic compounds. Arsenite and arsenate killed all the cells at the concentrations in which DMA and TMAO induced tetraploids and MMA, DMA, and TMAO induced mitotic arrest. This suggested that the absence of induction of tetraploids by arsenite and arsenate, was due to their cytotoxicity.

According to Risio and Rocci (1995), tetraploidy is an indicator of the risk to malignant transformation, while, tumor aneuploidy is a useful index of prognosis (Lazaris *et al.* 1995). In the skin cells from the patients of Bowen's disease, which was caused by arsenic, the rate of the tetraploid cells was increased (Argenyi *et al.* 1994). On the other hand, Schmitt

et al. (1995) reported that there was a strong relationship between aneuploidy and c-*erb* B-2 (proto-oncogene) expression. Bacus et al. (1990) assumed that aneuploidy preceded c-*erb* B-2 overexpression during carcinogenesis. It could be assumed from these that tetraploidy which was induced by arsenics might be one of the processes of carcinogenesis. Resolving the mechanisms of mitotic arrest and tetraploid formation caused by MMA, DMA, or TMAO may lead to the definition of the carcinogenetic mechanism of arsenic.

References

Argenyi ZB, Huston BM, Argenyi EE, Maillet MW, Hurt MA (1994) Large-cell acanthoma of the skin. Am J Derma 16:140–144

Bacus SS, Bacus JW, Slamon DJ, Press MF (1990) HER-2/neu oncogene expression and DNA ploidy analysis in breast cancer. Arch Pathol Lab Med 114:164–169

Beckma L, Nordenson I (1986) Interaction between some common genotic agents. Hum Hered 36(6):397–401

Bruno S, Ardelt B, Skierski JS, Traganos F, Darzynkiewicz Z (1992) Different effects of staurosporine, an inhibitor of protein kinases, on the cell cycle and chromatin structure of normal and leukemic lymphocytes. Cancer Res 51:470–473

Endo G, Kuroda K, Okamoto A, Horiguchi S (1992) Dimethylarsenic acid induces tetraploids in chinese hamster cells. Bull Environ Contam Toxicol 48:131–137

Felix MA, Cohen P, Karsenti E (1990) Cdc 2 H1 kinase is negatively regurated by a type 2A phosphatase in the Xenopus early embryonic cell cycle: evidence from the effects of okadaic acid. EMBO J 9:675–683

Fowler BA, Silbergeld EK (1989) Occupational disease: new workforces, new workplaces. Arch Environ Health 28:77–79

Gong J, Traganos F, Darzynkiewicz Z (1993) Simultaneous analysis of cell cycle kinetics at two different DNA ploidy levels based on DNA content and cyclin B measurements. Cancer Res 53:5096–5099

Hoyt MA, Totis L, Roberts BT (1991) S. cerevisiae genes required for cell cycle arrest in response to loss of microtubule function. Cell 66:507–511

Inoue Y, Kawabata K, Takahashi H, Endo G (1994) Determination of arsenic compounds using inductively coupled plasma mass spectrometry with ion chromatography. J Chromatogr A 675:149–154

International Agency for Research on Cancer (IARC) (1980) Arsenic and arsenic compounds. In: IARC monograph on the evaluation of the carcinogenic risk of chemicals to humans. Vol 23, International Agency for Research on Cancer. Lyon, pp 39–141

——— (1987) Arsenic and arsenic compounds. In: IARC monograph on the evaluation of the carcinogenic risks to humans. suppl. 7, 100–106 International Agency for Research on Cancer. Lyon, pp 285–293

Jacobson-Kram D, Montalbano D (1985) The reproductive effects assessment group's report on the mutagenicity of inornanic arsenic. Environ Mutagen 7:787–804

Kaise T, Horiguchi Y, Fukui S, Shiomi K, Chino M, Kikuchi T (1992) Acute toxicity and metabolism of arsenocholine in mice. Appl Organomet Chem 6:369–373

Kawabata K, Inoue Y, Takahashi H, Endo G (1994) Determination of arsenic species by inductively coupled plasma mass spectrometry ion chromatography. Appl Organomet Chem 8:245–248

Lazaris AC, Theodoropoulos GE, Anastassopoulos P, Nakopoulou L, Panoussopoulos D, Papadimitriou K (1995) Prognostic significance of p53 and c-erb B-2 immunohistochemical evaluation in colorectal adenocarcinoma. Histol Histopathol 10:661–668

Lee TC, Tanaka N, Lamb PW, Gilmer TM, Barret JC (1988) Induction of gene amplification by arsenic. Science 241(4861):79–81

National Institute for Occupational Safety and Health (NIOSH) (1976) Anon registry of toxic effects of chemical substance. NIOSH, Rockville, MD, pp 127, 129, 689

Nurse P (1990) Universal control mechanism regulating onset of M-phase. Nature (Lond.) 344:503–508

Ochi T, Kaise T, Oya-Ohta Y (1994) Glutathion plays different roles in the induction of the cytotoxic effects of inorganic and organic arsenic compounds in cultured BALB/c 3T3 cells. Experientia 50:115–120

Rieder CL, Palazzo RE (1992) Colcemid and the mitotic cycle. J. Cell Sci 102:387–392

Risio M, Rocci MP (1995) Intermediate biomarkers in the colorectal tumor progression. Tumori 81:16–18

Roberge M, Th'ng J, Hamaguchi J, Bradbury EM (1990) The topoisomerase II inhibitor VM-26 induces marked changes in histone H1 kinese activity, histones H1 and H3 phosphorylation, and chromosome condensation in G_2 phase and mitotic BHK cells. J Cell Biol 111:1753–1762

Schmitt FC, Figueiredo P, Lacerda M (1995) Expression of c-erb B-2 protein and DNA ploidy in brest carcinogenesis. Arch Pathol Lab Med 119 (9):815–820

Vahter M, Marafante E, Dencker L (1983) Metabolism of arsenobetaine in mice, rats and rabbits. Sci Total Environ 30:197–211

Vahter M, Norin H (1980) Metabolism of ^{74}As-labeled trivalent and pentavalent inorganic arcenic in mice. Environ Res 21:446–457

Vega L, Gonsebatt ME, Wegman PO (1995) Aneugenic effect of sodium arsenite on human lymphocytes in vitro: an indivisual susceptibility effect detected. Mutation Res 334:365–373

Wagner SL, Weswig P (1974) Arsenic in blood and urine of forest workers as indices of exposure to cacodylic acid. Arch Environ Health 28:77–79

Yager JW, Wiencke JK (1993) Enhancement of chromosomal damage by arsenic: implications for mechanism. Environ Health Perspectives 101 (Suppl. 3):79–82

Yamamoto S, Konishi Y, Matsuda T, Murai T, Shibata M, Yuasa I, Otani S, Kuroda K, Endo G, Fukushima S (1995) Cancer induction by an organic arsenic compound, dimethylarsinic acid (cacodylic acid), in F344/DuCrj rats after pretreatment with five carcinogens. Cancer Res 55:1271–1276

Yamauchi H, Yamamura Y (1984) Metabolism and excretion of orally administered dimethylarsinic acid in the hamster. Toxicol Apppl Pharmacol 74:134–140